Experimental Evaluation of the Effectiveness of Demineralized Bone Matrix and Collagenated Heterologous Bone Grafts Used Alone or in Combination with Platelet-Rich Fibrin on Bone Healing in Sinus Floor Augmentation

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Purpose: *The aim of this study was an experimental evaluation of the effectiveness of demineralized bone matrix (DBM) and collagenated heterologous bone graft (CHBG) used alone or in combination with platelet-rich fibrin on bone healing in sinus floor augmentation procedures.* ***Materials and Methods:*** *In this study, 36 New Zealand rabbits were used. The bilateral sinus elevation was performed, and 72 defects were obtained. The rabbit maxillary sinuses were divided into four groups according to the augmentation biomaterials obtained: demineralized bone matrix (Grafton DBM Putty, Osteotech; DBM group), DBM combined with platelet-rich fibrin (PRF; DBM + PRF group), collagenated heterologous bone graft (CHBG; Apatos Mix, OsteoBiol, Tecnoss; CHBG group), CHBG combined with PRF (CHBG + PRF group). All groups were sacrificed at 2, 4, and 8 weeks after surgery for histologic, histomorphometric, and immunohistochemical analyses.* ***Results:*** *The inflammatory reaction was moderate to intense at the second week in all groups and declined from 2 to 8 weeks. New bone formation was started at the second week and increased from 2 to 8 weeks in all groups. There was no significant difference in bone formation between the experimental groups that used PRF mixed graft material and control groups that used only graft material. The percentage of new bone formation showed a significant difference in DBM groups and DBM + PRF groups compared with other groups. There were osteoclasts around all the bone graft materials used, but the percentage of residual graft particles was significantly higher in CHBG groups and CHBG + PRF groups at the eighth week.* ***Conclusion:*** *There is no beneficial effect of the application of PRF in combination with demineralized bone matrix or collagenated heterologous bone graft on bone formation in sinus floor augmentation. The results of this study showed that both collagenated heterologous bone graft and demineralized bone matrix have osteoconductive properties, but demineralized bone matrix showed more bone formation than collagenated heterologous bone graft.* Int J Oral Maxillofac Implants2016;31:e24–e31. doi: 10.11607/jomi.4414

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In the atrophic posterior maxilla, implant placement is extremely dependent on bone augmentation of the maxillary sinus.[1–3] Several types of grafts involving autogenic, xenogeneic, allogeneic, and alloplastic materials have been used in this procedure, providing adequate bone height and volume.[4–6] Recently, the application of growth factors has drawn attention with their potency in the wound healing process. Platelet concentrates have been widely used in reconstructive surgery, as platelets are a natural source of growth factors, including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), and vascular endothelial growth factors (VEGF).[7,8] Platelet-rich fibrin (PRF) is a second-generation platelet concentrate that was developed in France by Choukroun et al[9] and is specifically for use in oral and maxillofacial surgery. The PRF clot is a strong autologous fibrin matrix, which performs a slow release of growth factors, with leukocytes and bioactive proteins existing inside.[10,11] It

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is considered that PRF may promote bone formation and bone repair in grafting procedures, so there have been several studies on the efficacy of PRF alone or combined with other bone substitutes.[12–19] To date, no study has been done on comparing the adjuvant effect of PRF in combination with a collagenated heterologous bone graft and a demineralized bone matrix. Collagenated heterologous bone graft is well integrated in the host site with its similarity to human bone and osteoconductivity, and it has been used in bone regeneration procedures recently.[20–23] Demineralized bone matrix (DBM) has osteoconductive and osteoinductive properties that contain much of the protein-based components native to bone that promote bone regeneration.[24–26] On the basis of the results obtained from the literature and numerous citation of studies related to evaluation of the potential of PRF to improve effectiveness of bone regeneration with various bone graft materials, it was hypothesized that the use of PRF in conjunction with DBM or CHBG might induce the rate of bone formation. In the present study, the authors applied PRF in combination with either collagenated heterologous bone graft or demineralized bone matrix to compare the potential effect of PRF with different grafting materials on bone formation after maxillary sinus grafting in a rabbit model. Even though these two were studied separately before, to the authors' knowledge, this is the first study to use DBM combined with PRF in comparison with CHBG combined with PRF.

MATERIALS AND METHODS

Study Design

This study followed the Declaration of Helsinki on medical protocol and ethics, and the regional Ethical Review Board of Gazi University Local Ethics Committee for Animal Experiments approved the study (G.Ü. B.30.2.GÜN.0.05.06.00/111-9172). Thirty-six male New Zealand white rabbits aged 3 to 3.5 months weighing 3 to 4 kg were used in this study. Both sinuses of each rabbit were filled with one of the bone substitutes and a combination of this material with PRF. A total of 72 sinuses were divided into four experimental groups according to the sinus grafting materials: collagenated heterologous bone graft group (sinus augmented with Apatos Mix [OsteoBiol, Tecnoss]), demineralized bone matrix group (sinus augmented with Grafton DBM Flex [Osteotech]), PRF + collagenated heterologous bone graft group, and PRF + DBM group. The rabbits were sacrificed at 2, 4, and 8 weeks after operation (12 animals in each interval).

Surgical Procedures

PRF Preparation. Autologous PRF was produced using the established protocol.[9,10,27] A 5-mL venous blood was drawn from each rabbit, and a 10-mL test tube (without anticoagulant) was centrifuged immediately at 3,000 rpm for 10 minutes in a laboratory centrifuge (Selecta Centronic-BL, J.P. SELECTA). After centrifugation, platelet-poor plasma at the top was collected by a syringe, and the PRF clot in the middle of the tube was separated from red corpuscles. It was cut into small pieces and mixed with DBM or CHBG to get a homogenous mass, improving the handling properties of graft material and providing graft stabilization.

Surgical Protocol. General anesthesia was induced by using an intramuscular injection of 50 mg/kg ketamine HCl (Ketasol %10, Richter Pharma) and 5 mg/kg Xylazine HCl (Rompun %2, Bayer); 0.5 mL of articaine HCl (Ultracain D-S, Sanofi Aventis) was injected locally at the periphery of the surgical field. Before the surgery, the surgical site was shaved and disinfected with a povidone-iodine topical antiseptic. The skin and subcutaneous tissues were incised in the middle of the nasal bone, and the periosteum was dissected bilaterally, exposing nasal bone and the nasoincisal suture line. Two round bone windows 8 mm in dimension located approximately 5 mm lateral to the midline and 10 mm anterior to the nasofrontal suture line were created bilaterally on the nasal bone with steel and diamond burs under sterile saline irrigation, leaving the sinus mucosa intact. The sinus membrane that moved back and forth with the respiratory rhythm was elevated very carefully to avoid any damage. Sinus elevators were used to gently elevate the membrane from the bony walls of the antrum to provide a compartment with a size of approximately $5 \times 5 \times 10$ mm. Only two small perforations of the sinus membrane had occurred, but they were sealed satisfactorily by resorbable collagen membrane (Evolution, OsteoBiol, Tecnoss). The sinus spaces were grafted with equal volumes of grafting materials such as CHBG, PRF + CHBG, DBM, or PRF + DBM to fill the created compartments. Material selection was done according to the blocked randomization method. Each of the experimental sites received a homogenous mixture of PRF clot with 125 mg of Grafton DBM Flex or Apatos Mix, and each of the control sites received only one of the two different bone substitutes lightly hydrated with saline. Both of the graft particles used alone or mixed with PRF were placed into the sinus space with a proper condensation. The bone defects were covered with resorbable collagen membrane. The periosteum and the skin were closed with a 4/0 resorbable suture (Pegasorb, Doğsan) after acquiring adequate hemostasis. The animals were given enrofloxacin (Baytril-K %5, Bayer), which was injected postsurgically for 3 days to prevent infection.

Histologic Preparations

The animals were sacrificed by intramuscular injection of ketamine HCl at 2 weeks (n = 12), 4 weeks (n = 12), and

8 weeks (n = 12). The four sites of the sinus were harvested, fixed in 10% formaldehyde solution for 48 to 72 hours, decalcified in 10% formic acid up to 3 weeks, and embedded in paraffin. Slides approximately 4 μm thick were taken from the specimens to perform hematoxylin-eosin staining for histologic evaluation and bone morphogenetic protein (BMP)-1 anticor for immunohistochemical evaluation and then examined by light microscopy.

Immunostaining Procedure

Slides were evaluated by immunohistochemical staining to detect the number of cells expressing BMP-1. These sections were deparaffinized in a 56.5°C oven overnight and using both xylene and ethanol baths. After rehydration, slides were treated with 3% H_2O_2 for 10 minutes and washed three times with tris-buffered saline (TBS) at pH 7.4 for 5 minutes. Slides were kept in Ultra V Block (ready-to-use-TA-060 UB, Lab Vision) for 5 minutes and incubated for 2 hours with primary antibodies (Rabbit polyclonal to BMP-1, ab118520, Abcam) at a dilution of 1:100 at room temperature. Slides were washed three times with TBS for 5 minutes and then incubated with secondary antibodies (ready-to-use TP-060-BN-Biotinylated Goat Anti-Polyvalent-Lab Vision) for 5 minutes. Slides were washed again three times with TBS and incubated with streptavidin peroxidase (TS-060-HR, Lab Vision) for 10 minutes at room temperature. After the final washing with TBS, slides were treated with diaminobenzidine tetrahydrochloride (DAB Plus Substrate Kit, Invitrogen) to obtain staining for immunoreaction. Slides were counterstained with hematoxylin-eosin (H&E), dehydrated in ethanol, cleared in xylene, and mounted in entellan. All slides were evaluated using a light microscope Leica DM 4000 B (Leica Microsystems). The expression of BMP-1 was evaluated as the number and percentage of positively stained cells. TBS was used instead of primary antibodies in control tissues and negative control slides. Osteoblasts and osteocytes with brown staining that were located around new bone areas were determined as positive. The expression of BMP was evaluated as the percentage of positively stained cells.

Histomorphometric Analysis

The histomorphometric data were obtained with captured histologic images at ×10 magnification using Leica Application System (LAS Version 4.2.0, Leica Microsystems) and analyzed by Leica QWin Plus v3.3.1 (Leica Microsystems). To calculate new bone formation, the photographs of five high-power fields were selected for each slide, and outlines of the newly formed bone, grafting materials, and total area were drawn. The areas formed were measured by an image analysis program and calculated as the percentage fraction of the new bone area and the graft particle area to total areas. All specimens were evaluated by one examiner who was blinded to the groups.

Statistical Analysis

Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) Statistics 20 Data Editor program. The frequency and percentage distribution of data were determined. The statistical differences between the groups were evaluated by the Kruskal-Wallis H test and Mann-Whitney *U* test at $P < .05$.

RESULTS

Histology

Biopsy specimens of the PRF and control sites showed that biomaterials used in these sites were biocompatible and distributed homogenously in the augmented area. By the second week, fibroblastic connective tissue was formed in the defect sides; also, there was slight bone regeneration at this week in all of the groups (Fig 1). In the CHBG and PRF + CHBG groups, new bone formation was in close contact with the particles, while in the DBM and PRF + DBM groups, it was seen in the maxillary host bone, far from the particles. At the second week, osteoblastic formation was more intense in the PRF + CHBG group than in the CHBG group (Fig 1). Collagen fibers built up, forming mature fibrous connective tissue in all groups at the fourth week. By the fourth week, the more advanced osteoblastic formation was seen from the periphery of the cavity to the center in the CHBG and PRF + CHBG groups and at the defect edges from the host bone to the particles in the DBM and PRF + DBM groups (Fig 2). At the eighth week, resorption of all graft particles was almost completed in the DBM and PRF + DBM groups, but residual particles were remaining in the CHBG and PRF + CHBG groups. By this week, there was advanced bone formation from the host bone to the center of the cavity forming bone islands in the DBM and PRF + DBM groups. In the CHBG and PRF + CHBG groups, trabeculated new bone was seen around graft particles as a thick band, and made anastomosis with the host bone (Fig 3).

Degrees of Inflammatory Reaction

The relative evaluation of inflammatory reaction was made according to the density of seven cell types (lymphocytes, plasmocytes, polymorphonuclear leukocytes, macrophages, necrosis, fibroblasts, giant-cell granulomas) by an experienced pathologist, and a numerical grade was assigned as follows: grade 0: absent, grade 1: mild, grade 2: moderate, grade 3: intense.

In the study and control groups, moderate or intense inflammation that was seen in the second week decreased progressively until the eighth week. In the DBM and PRF + DBM groups, a mild inflammatory response was observed at the eighth week, while there was no inflammatory reaction in the CHBG and PRF + CHBG groups (Table 1).

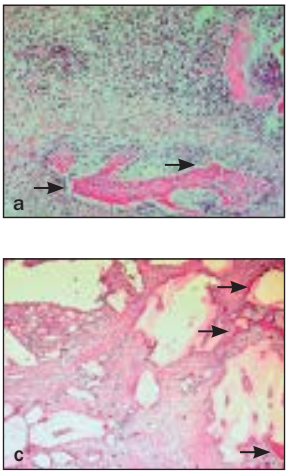

Fig 1 Histologic findings at the second week in *(a)* DBM-only group (H&E stain, ×200), *(b)* PRF + DBM group (H&E stain, ×200), *(c)* CHBG-only group (H&E stain, ×40), *(d)* PRF + CHBG combination group (H&E stain, ×100). Newly formed bone *(arrows)* is identified around or close by the implant materials.

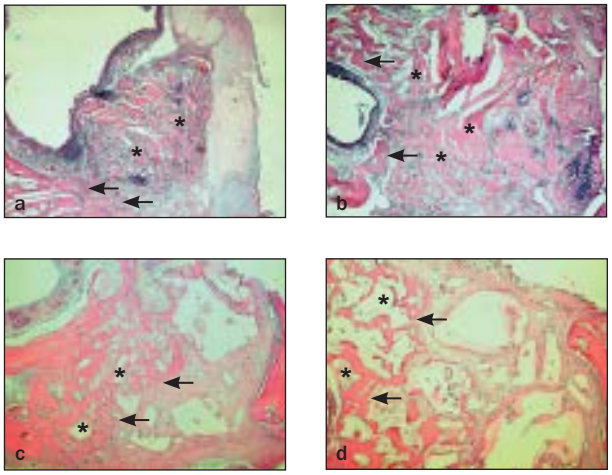

Fig 2 Histologic findings at the fourth week in *(a)* DBM-only group, *(b)* PRF + DBM group, *(c)* CHBG-only group, *(d)* PRF + CHBG combination group (H&E stain, ×40). Newly formed bone *(arrows)* is identified at the defect edges neighboring the implanted materials (*asterisks*).

Table 1 Inflammation Degree of Study and Control

	DBM groups	DBM + PRF groups	CHBG groups	CHBG + PRF groups
2nd week	3	3	1	2
4th week	3	3	1	1
8th week	1	1	0	0

0 = absent, 1 = mild, 2 = moderate, 3 = intense.

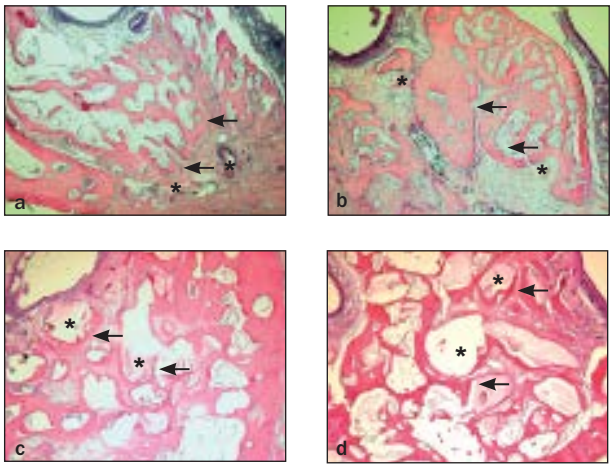

Fig 3 Histologic findings at the eighth week in *(a)* DBM-only group, *(b)* PRF + DBM group, *(c)* CHBG-only group, *(d)* PRF + CHBG combination group (H&E stain, ×40). *(a and b)* Advanced bone formation *(arrows)* was visible with resorption of almost all of the implanted material. *(c and d)* Anastomosing woven bone *(arrows)* was observed around the implant materials *(asterisks)*.

Histomorphometry

The areas of newly formed bone and residual graft particles at the second, fourth, and eighth weeks were measured in each group.

In the second week, the percentage of new bone formation in the DBM group was 11.2%, in the DBM + PRF group 9.45%, in the CHBG group 6.36%, and in the CHBG + PRF group 5.04%. In the fourth week, the DBM group was 29.5%, the DBM + PRF group 29.65%, the CHBG group 18.88%, and the CHBG + PRF group 11.56%. Finally, in the eighth week, the results were 41.02%, 38.64%, 33.21%, and 26.35%, respectively (Fig 4). The percentage of residual graft particles in the DBM group was 15.98%, in the DBM + PRF group 15.53%, in the CHBG group 21.35%, and in the CHBG + PRF group 21.39% in the second week. In the fourth week, the DBM group was 11.29%, the DBM + PRF group 8.77%, the CHBG group 18.64%, and the CHBG + PRF group 18.87%. Finally, in the eighth week , the results were 3.30%, 3.07%, 8.97%, and 13.92%, respectively (Fig 5).

The obtained data were analyzed; the percentage of newly formed bone area increased, and the graft site was reduced gradually by time. There was no statistical difference between the PRF-treated study groups compared with the graft material–only control groups in new bone formation and residual graft particle area ($P > .05$, Mann-Whitney U test). At the fourth week, there was a statistically significant difference between groups according to the graft material type in bone formation ($P = .007$). The newly formed bone area was higher in the DBM and DBM + PRF groups than in the CHBG and CHBG + PRF groups. At the eighth week, there was a statistically significant difference between

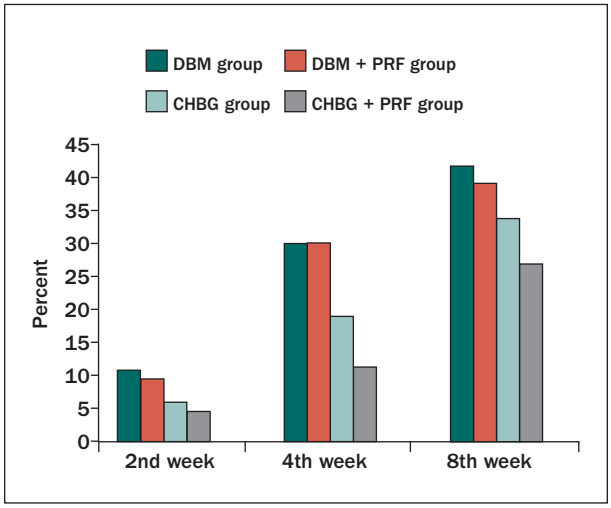

Fig 4 Quantitative analysis of bone formation area.

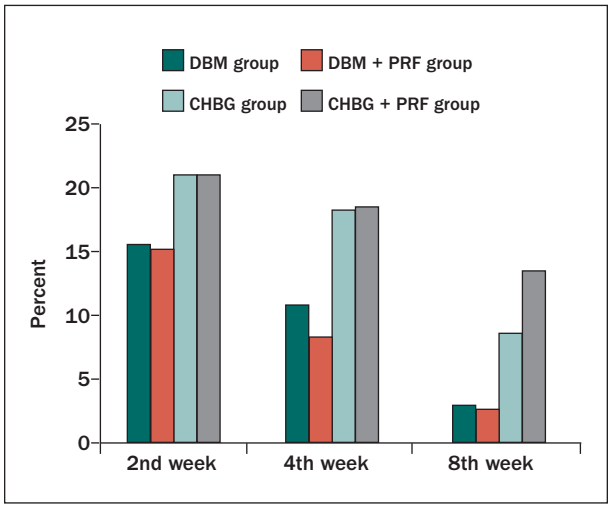

Fig 5 Quantitative analysis of residual graft area.

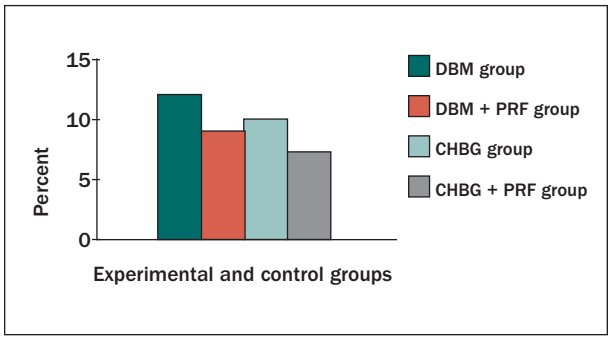

Fig 6 Quantitative analysis of BMP1 staining cells.

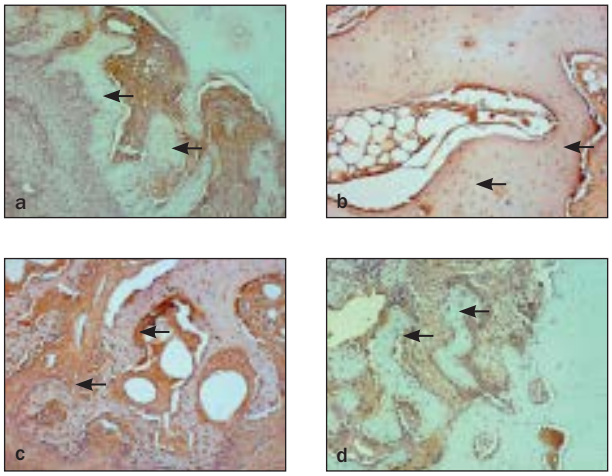

Fig 7 Immunohistochemical examinations: *(a)* DBM group, *(b)* DBM + PRF group, *(c)* CHBG group, *(d)* CHBG + PRF group stained with BMP1 antibody at the second week *(arrows)*.

groups according to the graft material type in newly formed bone ($P = .018$) and residual graft particle area ($P = .001$).

Immunohistochemistry

Immunohistochemical examination was performed, and the ratio of positively stained cells with antibodies to total number of cells was calculated. There were no statistical differences between PRF-treated study groups compared with graft material–only control groups in percentage of BMP-1 (+) cells (Fig 6; $P > .05$, Mann-Whitney *U* est). At the second week, there was a statistically significant difference between groups according to the graft material type ($P = .006$). The percentage of BMP-1 (+) cells was higher in the DBM and DBM + PRF groups than in the CHBG and CHBG + PRF groups (Fig 7).

DISCUSSION

Rehabilitation of a partially or totally edentulous maxilla may be complicated in implantology due to insufficient bone volume and the presence of the maxillary sinus.[28] Sinus floor augmentation has frequently been proposed as the optimal treatment option for achieving sufficient bone height and volume in the posterior maxilla.[29,30] Several grafting materials were developed for many years, such as DBM and collagenated heterologous bone grafts.[20,31] The maturation of these materials and their integration to the bone takes approximately 8 months.[32] Autologous growth factors have been used among various models of bone grafting aiming to enhance bone formation. Combinations of bone substitute material and growth factors appear to be ideal combinations to reduce the time interval. Currently, PRF is the most widely studied

and used concentrate in oral implantology; however, the studies evaluating the effect of PRF on new bone formation when used with bone graft materials are limited in the literature.[14,15,18,19]

This study aimed to assess the effectiveness of demineralized bone matrix, collagenated heterologous bone graft, and PRF when it is combined with either demineralized bone matrix or collagenated heterologous bone graft on bone formation in rabbit sinuses. As far as the authors know, this is the first study to use demineralized bone matrix combined with PRF in comparison with collagenated heterologous bone graft combined with PRF.

In experimental studies, the type of animal model varies according to the purpose of the study and is important for the clinical applicability.[33] The animal models must be close to some of the properties of human tissue as well as being economical enough. Rabbits are widely used in implant dentistry as an experimental model, due to their rapid bone healing that shortens the study period, as well as similar bone composition and sinus ventilation to humans.[34,35] Because of these advantages, rabbits were used in this experimental study.

In the experimental studies on sinus floor augmentation, different periods varying from 1 week to 6 months were seen.[36–38] A 2-week interval was chosen to assess osteoblastic proliferation and collagen production according to many studies that considered early new bone formation.[15,39] In previous studies, significant differences were seen in bone healing from 4 to 8 weeks, so a 4-week interval was chosen as the midperiod in order to see the beginning of the formation and calcification of the new bone tissue.[19,39,40] In two-stage sinus floor augmentation, implant placement is performed after an average 6- to 8-month time interval for integration of biomaterials to the bone in the healing process, and 8 weeks in rabbits can be compared with 6 to 8 months in humans.[33] According to these facts, this study was terminated at 8 weeks.

All the biomaterials placed in living tissue cause injury in the host tissue, and these materials are exposed to tissue response that includes some host reactions. These reactions are blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis or fibrous capsule formation.[41] These reactions occur within 2 to 3 weeks following the biomaterial placement. The inflammatory response of biocompatible materials is usually terminated in a short period. Understanding the duration of inflammation and foreign body reaction caused by a biomaterial is important in the evaluation of biocompatibility.[42] Inflammation was evaluated to assess the biocompatibility of all materials used in this study, including PRF, which has strong antiinflammatory effects.[43] According to histologic examination, moderate and intense inflammation, which were seen at the second week, decreased progressively in all groups, and mild or no inflammatory response was observed at the eighth week. In contrast to some studies, it was observed that PRF cannot regulate the inflammatory response.[11,44] More intense inflammation was seen in the groups that used DBM than those that used CHBG. This result was consistent with the results of Sicca et al,[39] who reported that inflammatory response occurs around the heterogeneous organic matrix of bone. In this study, a mild inflammatory response was seen in groups that used CHBG, confirming the findings of Nannmark and Sennerby.[20] Biomaterials that were used in this study did not cause any foreign body reaction as in previous clinical and experimental studies.[20,22,45,46]

In many studies that determine the bone healing mechanisms of the graft materials in sinus floor augmentation procedures, newly formed connective tissue and the formation of vascular and bone tissue were evaluated histologically.[19,23,34,38] The histologic findings of the present study corroborated Kim et al,[19] who reported that PRF increases osteoblastic proliferation in early bone formation, indicating that osteoblastic proliferation was seen in the PRF combined with CHBG group more than the CHBG-only group at the second week, and PRF may have a positive effect on bone healing when combined with CHBG. According to the literature, the graft materials used in this study provide osteoconductivity in different ways; CHBG was reported to have a good degree of integration without the formation of connective tissue or fibrous tissue between the particles and newly formed bone, acting as a scaffold,[22,47,48] as DBM is reported to be osteoconductive by forming new bone in fibrous tissue with a similar process of chronic inflammation.[49] In this study, the new bone formation occurred around CHBG particles toward the center of the defect edge by connecting with each other, as it was observed far from the DBM particles close to the host bone with a chronic inflammatory reaction.

Ideal bone grafts should encourage new bone formation by absorption of the particle replacement by newly formed bone tissue.[50] The degradation time of the biomaterials is important as it affects the space maintenance in sinus floor augmentation.[51] In the present study, slow degeneration of the collagenated heterologous bone graft particles was observed in both the groups that used CHBG, which was consistent with some studies.[47,52,53] On the other hand, in the DBM group, remaining graft particles decreased over time, and at the end of the eighth week, the resorption of almost all the particles was observed, leading most of the field filled with newly formed bone tissue. In contrast with some studies,[24,47,54] this result indicated that DBM shows more rapid resorption and new bone formation.

In the literature, there are a few studies[14,15,18,19] about effects of PRF on bone healing. Some of these studies[14,17,19] concluded that PRF has positive effects on bone

healing; other studies[15,16,55] have reported that PRF has no effects on bone healing. Bensaïd et al[56] established the optimal fibrinogen concentration and thrombin activity for transplantation of mesenchymal stem cells in vitro and reported that 100 IU/mL thrombin activity and 18 mg/mL fibrinogen concentration are ideal for the proliferation and spreading of these cells. It has also been reported that higher concentrations prevented the spread and proliferation of cells.[56] In the present study, the effectiveness of PRF on bone healing was not statistically significant between the study and control groups. These data can be explained by the effect of PRF on bone healing being associated with the concentration of the material, and it depends on the concentration of the PRF volume achieved. Pripatnanont et al[18] reported that PRF has a positive effect on bone formation when used alone or in combination with autogenous bone but no significant effect when used with deproteinized bovine bone and concluded that PRF has no direct benefit in the absence of living osteogenic cells. The inefficacy of PRF observed in this study is associated with the absence of viable cells in osteogenic medium, in addition to the aforementioned theory.

Various antibodies are used in the immunohistochemical evaluation of bone reconstruction procedures or the biomaterials that are used in these procedures considering new bone formation.[57,58] According to the immunohistochemical examinations in the present study, BMP1 expression was seen in all groups. The pattern of immunohistochemical staining was found to be similar to the findings of Silva et al,[59] as the expression of BMP1 was observed in osteoprogenitor cells and the surrounding extracellular matrix. The expression of BMP1 was significantly more intense in groups that used DBM than CHBG groups at the second week, and it is associated with the increased new bone formation that was seen at the fourth and eighth weeks in groups that used DBM compared with CHBG groups. Despite the differences in the percentage of BMP1(+) cells, there was no statistically significant difference between the study and control groups. This result is consistent with the results of histologic and histomorphometric methods used in this study.

CONCLUSIONS

The results of this study suggest that PRF may increase osteoblastic proliferation when used with collagenated heterologous bone graft in early bone formation. Also, it is indicated that DBM particles resorb more than CHBG particles and that DBM shows more rapid bone formation than CHBG, but their combination with PRF does not show a significant effect on bone regeneration. Further large-scale studies are needed to confirm the results of this study.

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